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Lessons in Membrane Engineering for Octanoic Acid Production from Environmental *Escherichia coli* Isolates

Abstract

Fermentative production of many attractive biorenewable fuels and chemicals is limited by product toxicity in the form of damage to the microbial cell membrane. Metabolic engineering of the production organism can help mitigate this problem, but there is a need for identification and prioritization of the most effective engineering targets. Here, we use a set of previously characterized environmental *Escherichia coli* isolates with high tolerance and production of octanoic acid, a model membrane-damaging biorenewable product, as a case study for identifying and prioritizing membrane engineering strategies. This characterization identified differences in the membrane lipid composition, fluidity, integrity, and cell surface hydrophobicity from those of the lab strain MG1655. Consistent with previous publications, decreased membrane fluidity was associated with increased fatty acid production ability. Maintenance of high membrane integrity or longer membrane lipids seemed to be of less importance than fluidity. Cell surface hydrophobicity was also directly associated with fatty acid production titers, with the strength of this association demonstrated by plasmid-based expression of the multiple stress resistance outer membrane protein BhsA. This expression of bhsA was effective in altering hydrophobicity, but the direction and magnitude of the change differed between strains. Thus, additional strategies are needed to reliably engineer cell surface hydrophobicity. This work demonstrates the ability of environmental microbiological studies to impact the metabolic engineering design-build-test-learn cycle and possibly increase the economic viability of fermentative bioprocesses.

Keywords

bhsA, cyclic, fatty acids, fluidity, hydrophobicity, inhibition, integrity, membrane, tolerance, unsaturated

Disciplines

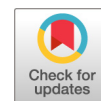
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Lessons in Membrane Engineering for Octanoic Acid Production from Environmental *Escherichia coli* Isolates

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ABSTRACT Fermentative production of many attractive biorenewable fuels and chemicals is limited by product toxicity in the form of damage to the microbial cell membrane. Metabolic engineering of the production organism can help mitigate this problem, but there is a need for identification and prioritization of the most effective engineering targets. Here, we use a set of previously characterized environmental *Escherichia coli* isolates with high tolerance and production of octanoic acid, a model membrane-damaging biorenewable product, as a case study for identifying and prioritizing membrane engineering strategies. This characterization identified differences in the membrane lipid composition, fluidity, integrity, and cell surface hydrophobicity from those of the lab strain MG1655. Consistent with previous publications, decreased membrane fluidity was associated with increased fatty acid production ability. Maintenance of high membrane integrity or longer membrane lipids seemed to be of less importance than fluidity. Cell surface hydrophobicity was also directly associated with fatty acid production titers, with the strength of this association demonstrated by plasmid-based expression of the multiple stress resistance outer membrane protein BhsA. This expression of *bhsA* was effective in altering hydrophobicity, but the direction and magnitude of the change differed between strains. Thus, additional strategies are needed to reliably engineer cell surface hydrophobicity. This work demonstrates the ability of environmental microbiological studies to impact the metabolic engineering design-build-test-learn cycle and possibly increase the economic viability of fermentative bioprocesses.

IMPORTANCE The production of bulk fuels and chemicals in a bio-based fermentation process requires high product titers. This is often difficult to achieve, because many of the target molecules damage the membrane of the microbial cell factory. Engineering the composition of the membrane in order to decrease its vulnerability to this damage has proven to be an effective strategy for improving bioproduction, but additional strategies and engineering targets are needed. Here, we studied a small set of environmental *Escherichia coli* isolates that have higher production titers of octanoic acid, a model biorenewable chemical, than those of the lab strain MG1655. We found that membrane fluidity and cell surface hydrophobicity are strongly associated with improved octanoic acid production. Fewer genetic modification strategies have been demonstrated for tuning hydrophobicity relative to fluidity, leading to the conclusion that there is a need for expanding hydrophobicity engineering strategies in *E. coli*.

KEYWORDS *bhsA*, cyclic, fatty acids, fluidity, hydrophobicity, inhibition, integrity, membrane, tolerance, unsaturated

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Throughout the vast majority of human history, transportation energy and commodity goods have been obtained from woody biomass, crops, and animals (1–3). However, in the past century, petroleum has become the predominant source of carbon and energy for the production of fuels and chemicals, with a variety of undesirable outcomes (4–6). The fermentative production of bulk fuels and chemicals, such as ethanol, lactic acid, acetone, and butanol, for example, was commercially viable prior to the establishment of the petrochemical industry (7, 8). However, the economically viable production of bio-based fuels and chemicals that could displace petroleum-derived molecules still remains challenging (6).

One of the limitations of fermentative production of bulk fuels and chemicals is toxicity, both in terms of the product molecules and the biomass-derived carbon streams (9, 10). This toxicity can be addressed through *in situ* product removal (11–14) and/or increasing the robustness of the microbial biocatalyst (15–18). Design strategies for robust biocatalysts can be based on known mechanisms of inhibition and tolerance (19–21). Implementation of the “learn” step of the design-build-test-learn cycle (22, 23) can provide inspiration for such rational design strategies. This learning can come from the characterization of evolved strains (21) or wild populations (24).

Fatty acids have a large and increasing industrial demand due to their wide range of applications. Short-chain fatty acids are directly used as a food preservative (25, 26) and dietary supplement (27). Fatty acids also can be used as precursors for a variety of industrial chemicals, such as alkanes (28, 29), α -olefins (30, 31), and fatty acid methyl or ethyl esters (32). Extensive work has been done to improve the fermentative production of fatty acids (5, 31, 33–35). However, as with many other desirable biorenewable products (36, 37), fatty acid toxicity to microbes (9, 10, 19, 38) limits product titers and thus negatively impacts the economic viability of bio-based fatty acid production. Substantial progress has been made in addressing the cytotoxicity of fatty acids in *Escherichia coli* (9, 18, 33), with a variety of studies concluding that membrane damage is the major mechanism of fatty acid toxicity (9, 10, 18, 19, 38). This membrane damage corresponds to a decrease in membrane integrity (9) and perturbation of membrane fluidity (9, 18). Engineering the cell membrane composition (e.g., lipid distribution) to mitigate the damage imposed by fatty acids (33, 39), enabling the production of trans-unsaturated fatty acids (16), and altering the phospholipid head distribution (40) have proven effective for increasing fatty acid tolerance and/or production. Despite this progress, further increases in fatty acid titer are desirable, particularly in terms of short-chain fatty acids, such as octanoic acid (C_8).

A previously described collection of environmental *E. coli* isolates was found to have wide variation in membrane properties, such as hydrophobicity and zeta potential (41). Here, we characterized these strains for their ability to tolerate and produce octanoic acid. This characterization identified membrane properties that are promising additions to the arsenal of strategies for improving microbial robustness for increasing the production of biorenewable fuels and chemicals.

RESULTS

Environmental isolates show increased fatty acid tolerance and production. It has previously been demonstrated that short-chain fatty acids are inhibitory to *E. coli*, having a detrimental impact on both growth and fatty acid production (9, 18, 42). Several *E. coli* strains were previously isolated from Squaw Creek in Ames, IA, and characterized in terms of various cell surface properties and phylotype (Table 1) (41). Given that these environmental isolates have previously demonstrated an ability to survive and propagate under harsh environmental conditions, we proposed that these environmental isolates may be able to provide insight into design strategies for developing robust microbial cell factories for short-chain fatty acid production. Fifteen of these previously characterized environmental isolates were compared to the standard K-12 lab strain MG1655 in terms of short-chain fatty acid sensitivity and production (Table 2). Specifically, strains were grown in morpholinepropanesulfonic acid (MOPS) medium with 2.0 wt% dextrose at 37°C with and without 10 mM C_8 at pH 7.0.

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Genotype or description ^a	Phylogroup	Reference or source
Strains			
MG1655	K-12 wild type	A	Wild type
9, 18, 284	Environmental <i>E. coli</i> isolates from Squaw Creek, Ames, IA	A/C	42
8, 13, 28, 48, 57, 236, 248		B1	
6, 32, 44, 83		D/E	
292		Unknown	
Plasmids			
pJMYEEI82564	pTrc-EEI82564 thioesterase (TE10) from <i>Anaerococcus tetradus</i> , Amp ^r		18
pACYC184-Kan- <i>bhsA</i>	pACYC184-Kan harboring <i>bhsA</i> , Kan ^r		This work

^aAmp^r, ampicillin resistance; Kan^r, kanamycin resistance.

Nearly all of the strains showed less than 20% inhibition relative to growth in the absence of C₈. Note that environmental isolate 6 showed a very high C₈ sensitivity, with approximately 90% inhibition. All of the environmental isolates, except for strains 48 and 236, showed significantly ($P < 0.001$) less inhibition by 10 mM C₈ than did MG1655 (Table 2).

Given these promising results regarding fatty acid tolerance, we then characterized short-chain fatty acid production for a smaller set of these environmental isolates via expression of the *Anaerococcus tetradus* thioesterase. This set was selected so that there was at least one isolate from each of the phylogroups. Fatty acid production was characterized for seven of these environmental isolates and MG1655 in MOPS medium with 2.0 wt% dextrose at 30°C (Table 2). All of the environmental isolates had significantly higher fatty acid titers than MG1655. Specifically, MG1655 produced 56.4 ± 0.8 mg/liter, while all of the isolates produced at least 145 mg/liter. Note that the highest observed titer of 251 mg/liter corresponds to less than 2 mM fatty acids, which is substantially lower than the 10 mM concentration used for the toxicity tests.

All of the environmental isolates also showed approximately 5-fold higher biomass production during fatty acid production than did MG1655. Productivity values over the first 24 h ranged from 1.1 mg/liter/optical density at 550 nm (OD₅₅₀)/h for isolate 44 to

TABLE 2 Environmental isolates have decreased fatty acid sensitivity and increased fatty acid production relative to MG1655^a

Strain	OD ₅₅₀ at 8 h, 37°C by C ₈ concn (mM)		% decrease in OD ₅₅₀	Fatty acid titer (mg/liter) by productivity time (h)		OD ₅₅₀ during fatty acid production by productivity time (h)	
	0	10		24	48	24	48
MG1655	2.0 ± 0.1	1.69 ± 0.01	15.5 ± 0.8	52.7 ± 0.8	56.4 ± 0.8	0.59 ± 0.01	0.58 ± 0.01
6	1.7 ± 0.1	0.145 ± 0.03	90 ± 20				
8	1.94 ± 0.01	1.70 ± 0.02	12.4 ± 0.2	235 ± 7 ^b	213 ± 5 ^b	3.7 ± 0.2 ^b	3.92 ± 0.05 ^b
9	1.8 ± 0.0	1.69 ± 0.02	6.1 ± 0.1				
13	1.8 ± 0.1	1.7 ± 0.0	5.6 ± 0.3				
18	1.84 ± 0.02	1.67 ± 0.02	9.2 ± 0.2	144 ± 2 ^b	148 ± 3 ^b	3.00 ± 0.02 ^b	3.64 ± 0.06 ^b
28	1.80 ± 0.04	1.69 ± 0.01	6.1 ± 0.1				
32	1.86 ± 0.07	1.640 ± 0.002	11.8 ± 0.5				
44	1.83 ± 0.05	1.61 ± 0.04	12.0 ± 0.4	101 ± 1 ^b	200 ± 10 ^b	3.8 ± 0.1 ^b	4.0 ± 0.1 ^b
48	2.07 ± 0.02	1.77 ± 0.01	14.5 ± 0.2 ^c	200 ± 20 ^b	228 ± 9 ^b	3.6 ± 0.1 ^b	4.01 ± 0.02 ^b
57	1.68 ± 0.05	1.56 ± 0.01	7.1 ± 0.2				
83	1.86 ± 0.05	1.62 ± 0.06	12.9 ± 0.6	245 ± 5 ^b	251 ± 3 ^b	3.6 ± 0.1 ^b	3.98 ± 0.01 ^b
236	2.04 ± 0.01	1.72 ± 0.01	15.7 ± 0.1 ^c	220 ± 10 ^b	216 ± 6 ^b	3.7 ± 0.1 ^b	3.8 ± 0.1 ^b
248	1.92 ± 0.06	1.75 ± 0.05	8.9 ± 0.4				
284	1.90 ± 0.06	1.71 ± 0.02	10.0 ± 0.3				
292	1.96 ± 0.03	1.73 ± 0.07	11.7 ± 0.5	245 ± 5 ^b	251 ± 2 ^b	2.98 ± 0.03 ^b	2.84 ± 0.04 ^b

^aStrains were grown in MOPS minimal medium with 2.0 wt% dextrose, with or without 10 mM C₈ at pH 7.00 ± 0.05. For fatty acid production, strains expressed the TE10 thioesterase from *Anaerococcus tetradus* via the pJMYEEI82564 plasmid induced with 1.0 mM IPTG. Growth was assessed at 37°C, and production was assessed at 30°C. Productivity values are given in Table S1.

^bSignificantly ($P \leq 0.0001$) different fatty acid titer or OD₅₅₀ during fatty acid production relative to MG1655.

^cEnvironmental isolates whose percent decrease in OD₅₅₀ was not significantly different ($P > 0.05$) from MG1655.

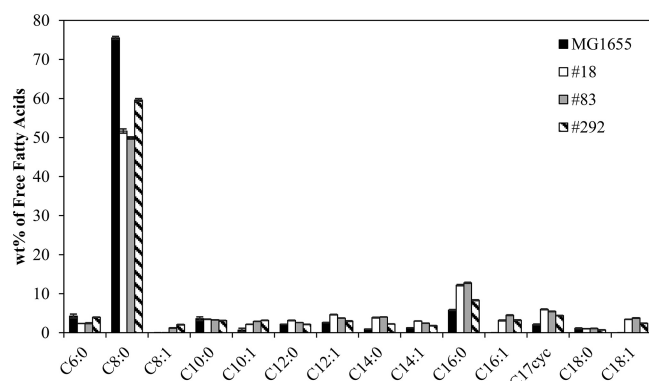


FIG 1 The focal environmental *E. coli* isolates have decreased chain length specificity among the free fatty acid pool relative to that of MG1655. All strains contain the pJMYEE182564 plasmid encoding the *A. tetradis* thioesterase. Strains were grown for 48 h in MOPS mineral salts medium with 2.0% (wt/vol) dextrose at 30°C, initial pH 7.0, and at 200 rpm with 1.0 mM IPTG. Values are the average of three biological replicates, with error bars indicating one standard deviation.

3.7 mg/liter/OD₅₅₀/h for MG1655 (see Table S1 in the supplemental material). The high productivity of MG1655 is largely due to the very low OD attained during fatty acid production. These results provide further motivation for characterizing these isolates in order to identify design strategies for increasing fatty acid production in industrial strains. We selected three environmental isolates for further characterization. Isolates 292 and 83 had the two highest 24-h and 48-h productivity values compared to the other environmental isolates. Isolate 18 had the lowest 48-h productivity value compared to those of the other environmental isolates.

Measurement of the free fatty acid chain length distribution indicates that the wild-type lab strain MG1655 shows increased specificity for C_{8:0} fatty acids relative to that of the environmental isolates (Fig. 1). Specifically, the pool of free fatty acids produced by MG1655 contained more than 75 wt% C₈. The free fatty acid pools produced by the environmental isolates contained between 50 and 60 wt% C₈, with a shifting of the distribution toward chain lengths of 14 or more carbons.

Identification of design strategies from the membrane lipid distribution. Damage to the microbial membrane is a key component of fatty acid toxicity (9, 19, 38), and changes to the membrane composition have been associated with increased fatty acid tolerance and production (16, 18, 33, 39, 40). Characterization of the membrane of each of our selected focal *E. coli* strains during fatty acid challenge and during fatty acid production supports the contribution of the lipid tail distribution to differences in strain robustness (Table 3). A few differences in membrane lipid tail distribution were observed between the environmental isolates and MG1655, regardless of the presence or production of octanoic acid (Table 3). Specifically, under all conditions, strain 83 had an above-average abundance of C_{12:0}, while MG1655 had below-average abundance. Also, each of the environmental isolates showed increased abundance of C_{12:1} during fatty acid production relative to that of the nonproduction condition. This trend was not observed for MG1655.

It has previously been demonstrated that increasing the average fatty acid chain length in the membrane can improve fatty acid production (39). Each of the environmental isolates characterized here showed a decrease in average lipid length during fatty acid production relative to that under the nonproduction condition (Table 3; see also Table S2 in the supplemental material). While MG1655 also showed a decrease, the magnitude was smaller. Specifically, MG1655 had a 1% decrease in average lipid length under the 24-h condition relative to that under the 0 mM control, while each of the environmental isolates had a decrease of 3% or more (Table S2). The percent decrease in the average lipid length significantly differed for MG1655 and isolate 292 ($P < 0.005$).

The relative degree of unsaturation is also known to be a key metric of membrane lipid composition (9, 18, 33). Previous reports have described an increase in the relative

TABLE 3 Membrane fatty acid tail composition differs among MG1655 and the environmental isolates during C₈ challenge and fatty acid production^a

	MG1655				#18				#83				#292			
	control	+30 mM	24 hr	48 hr	control	+30 mM	24 hr	48 hr	control	+30 mM	24 hr	48 hr	control	+30 mM	24 hr	48 hr
C12:0	0.5	1.7	0	0	0	7	3.28	2.4	3.9	4.3	4	3.97	0.3	1.03	4.02	4.2
C12:1	0	0	0	0	0	0	0.8	0.7	0	0	2.1	2.56	0	0	1.6	1.79
C14:0	4.6	3.9	3.9	5	6	4.9	11.1	9.5	12	7.1	11.2	10.7	4.3	4.2	9.4	8.9
C14:1	0	0	0	2.7	0	0	5.8	5.4	0	0	6.27	7	0	0	6.5	7.01
C16:0	47	49	54	55	46	37.3	37.4	37.3	37	39.1	33.9	30.5	42	39.6	33.4	30.6
C16:1	15.5	11.1	3.9	4.6	20.9	23.3	7.8	7.26	11.6	17	11.9	13.2	24.7	26.9	12.35	13.4
C17cyc	13.4	11	27	19.2	7	5.3	20.5	22.1	14.4	10.1	16.5	16.1	8.2	5	19	18.9
C18:0	1.11	2.2	2	2.9	2.8	3.82	1.1	2.8	0.8	2.6	2.113	2.4	0.94	1.852	1.8	2.06
C18:1	16	20	5.8	7	17.5	17.8	9.2	9.1	18.9	19	9.9	11.4	19.6	21.1	10	10.97
C19cyc	1.9	1.8	2.1	2.1	0	0.5	2.4	3.1	1.8	0.85	2	2.3	0.6	0.4	1.7741	1.91
average length	16.27	16.3	16.1	16.1	16.3	16.1	15.77	15.9	16.04	16.14	15.6	15.708	16.33	16.343	15.73	15.73
% unsaturated	31	30	10	14	39	41.4	23.6	22.7	30.4	35.9	30.2	34.1	44.4	48.2	31	33.5
% cyclic	15.4	12.1	30	20.2	7	5.8	23	24.6	16.3	10.9	18.5	18.4	8.8	5	20.8	20.8

^aStrains were assessed in MOPS plus 2.0 wt% dextrose at 30°C in shake flasks at 200 rpm. Cells assessed for the control and plus 30 mM conditions do not encode the thioesterase. All cultures had an initial pH of 7.0. For the control and plus 30 mM experiments, cells were harvested at mid-log phase, corresponding to an OD₅₅₀ of approximately 1.0. Numerical values are the average of three biological replicates, with the number of significant digits shown being selected based on the value of the standard deviation. Shading indicates the value relative to the median for each row according to the 95% confidence intervals. Values shaded in red are smaller than the median, and values shaded in green are larger than the median.

abundance of unsaturated C₁₆ and C₁₈ lipids in the membrane during fatty acid production by a K-12 *E. coli* strain (33). However, the characterization reported here observed a significant decrease in the relative abundance of unsaturated fatty acids in the membrane of MG1655 during fatty acid production (Table 3; see also Table S2 in the supplemental material). While the environmental isolates also showed, in general, a decrease in the abundance of unsaturated lipids during fatty acid production relative to that under the control condition, this change was significantly ($P < 0.005$) smaller for all of the isolates than for MG1655 (Table S2). It should also be noted that the abundance of unsaturated membrane lipids was lower during fatty acid production for MG1655 than for the environmental isolates.

The opposite trend was observed for the relative abundance of cyclic fatty acids, in that their abundance generally increased in all strains during fatty acid production. For isolate 18, the percent change in cyclic fatty acid content under the 24-h condition relative to that under the control was significantly larger than for MG1655 (Table S2). For isolate 83, this change was significantly smaller than for MG1655.

A broad interpretation of these trends is that changes in the MG1655 membrane during fatty acid production mainly consisted of changes in the abundance of unsaturated and cyclic fatty acids and not length. Contrastingly, changes in the membranes of the environmental isolates had more of an impact on length and less of an impact on unsaturated lipid content.

In addition to the membrane composition metrics described above, the C_{16:1}/C_{16:0} ratios differ between these strains (Fig. 2). The MG1655 membrane showed a significant decrease in this ratio during challenge with 30 mM C₈ relative to that with the untreated control, decreasing from 0.32 to 0.23. In contrast, each of the environmental isolates showed an increase in this ratio. For example, for strain 18, this ratio increased from 0.46 to 0.63 ($P < 0.0006$). During fatty acid production, the three environmental isolates all had a significantly higher C_{16:1}/C_{16:0} ratio than the MG1655 strain ($P < 0.0006$). At 24 h, this ratio was 0.07 for MG1655 but was 0.20 for strain 18, 0.35 for strain 83, and 0.37 for strain 292 (Fig. 2). Previous membrane engineering efforts have aimed to engineer the membrane composition in terms of average lipid length and abundance of unsaturated lipids. Our results suggest that it may also be useful to target the C_{16:1}/C_{16:0} ratio.

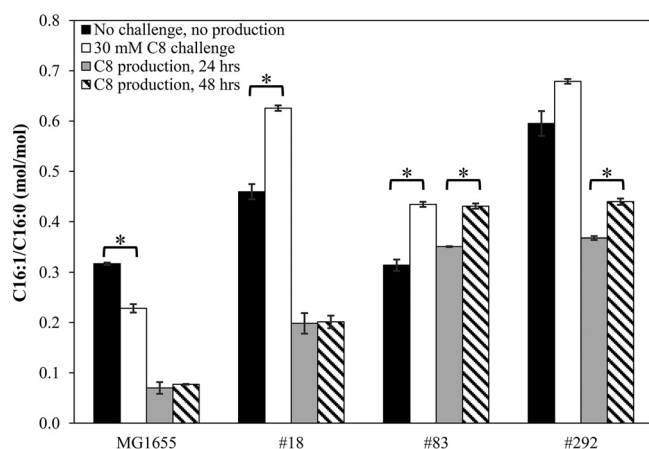


FIG 2 Increased growth during C_8 challenge and increased C_8 production by environmental isolates 83 and 292 are associated with an increase in $C_{16:1}$ relative to $C_{16:0}$ in the presence of C_8 . All strains were grown in MOPS minimal medium containing 2.0 wt% dextrose at 30°C with an initial pH 7.0. Values are the average of three biological replicates, with error bars indicating one standard deviation. *, significant difference ($P \leq 0.0006$), selected using the Bonferroni correction.

Decreased membrane fluidity is associated with increased fatty acid production. Membrane damage is generally considered a key mechanism of *E. coli* inhibition during challenge with or the production of short-chain fatty acids, along with many other biorenewable fuels and chemicals (9, 16, 18). The membrane composition can be quantified by metrics, such as the lipid tail distribution, as described above. However, the membrane also consists of many proteins, sugars, and phospholipid head groups. Metrics such as membrane fluidity and integrity reflect the overall function and state of the membrane.

Maintenance of appropriate membrane fluidity is vital to membrane function. In general, membrane fluidity trends with the relative abundance of unsaturated lipids (43), though other membrane components can also contribute to this property (16, 44, 45). Membrane fluidity was measured via cell membrane fluorescence polarization, where increased polarization corresponds to decreased membrane fluidity (9, 46). Our previous studies have shown that the membrane fluorescence polarization of MG1655 decreased substantially during challenge with exogenous C_8 without prior adaptation (9). However, cells that had 3 h of prior exposure to 30 mM C_8 were able to maintain a polarization of approximately 0.3 during exogenous C_8 challenge. These prior results demonstrate that free fatty acids could change membrane fluorescence polarization, but changes in the membrane composition could make the cell membrane more resistant to this effect. Here, membrane fluorescence polarization was assessed for the fatty acid-producing strains at 24 and 48 h (Fig. 3A). At 24 h, isolates 18 and 292 had significantly ($P < 0.05$) increased polarization compared to MG1655. At 48 h, all three isolates had significantly increased polarization compared to MG1655. The increased polarization during fatty acid production for the environmental isolates compared to MG1655 indicates that the isolates have a lower membrane fluidity than MG1655.

Previous studies of an evolved *E. coli* strain and an *E. coli* strain engineered to produce a novel membrane lipid tail also reported that decreased membrane fluidity was associated with increased fatty acid production (16, 18). The isolate characterization described here provides further evidence that decreasing membrane fluidity is a promising strategy for improving short-chain fatty acid tolerance and production. Surprisingly, the isolates all had a higher abundance of unsaturated lipids than did MG1655 (Table 3), leading to the expectation of higher fluidity. Thus, differences in fluidity cannot be solely attributed to the relative abundance of unsaturated fatty acids in the membrane.

The isolates also differ from MG1655 in that their membrane fluorescence polarization value significantly ($P < 0.05$) increased from 24 h to 48 h (Fig. 3A). These results

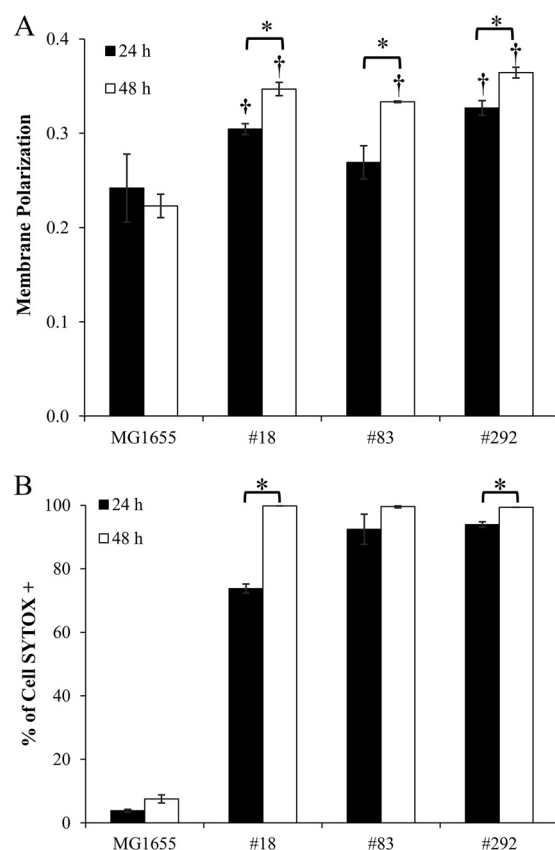


FIG 3 Membrane fluidity and integrity change during fatty acid production and differ for the environmental isolates relative to those in MG1655. All values are the average of three biological replicates, with error bars indicating one standard deviation. (A) Membrane fluorescence polarization, which is inversely proportional to membrane fluidity, significantly increased in the environmental isolates during the course of C_8 production. (B) Membrane integrity, which is inversely proportional to SYTOX permeability, significantly decreased during the course of fatty acid production in environmental isolates 18 and 292. *, significant difference ($P \leq 0.05$) between 24 h and 48 h for a single strain. †, significant difference ($P \leq 0.05$) in the membrane polarization between MG1655 and environmental isolates at 24 h or 48 h.

indicate that the environmental isolates are able to further modulate their membrane fluidity. For isolates 83 and 292, this further increase in polarization is surprising, given the observed significant ($P < 0.05$) increase in the percentage of unsaturated fatty acids (Table 3) and the $C_{16:1}/C_{16:0}$ ratio (Fig. 2). This provides further demonstration that fluidity is not solely a function of unsaturated fatty acid content. Isolate 18 did not have a significant increase in the percentage of unsaturated fatty acids or $C_{16:1}/C_{16:0}$ ratio. Instead, isolate 18 showed a significant ($P = 0.041$) increase in the average lipid length. Thus, the environmental isolates might be using distinct strategies to control membrane fluidity during fatty acid production.

Investigation of membrane integrity during fatty acid production. Decreased membrane integrity is a widespread problem during microbial production of fuels and chemicals (15, 16, 18, 38, 40). Membrane integrity can be quantified via the permeability of cells to the SYTOX nucleic acid dye (33). At both time points during fatty acid production, MG1655 had dramatically decreased SYTOX permeability relative to that of the environmental isolates (Fig. 3B). This could be due to the very low fatty acid titer of MG1655 (Table 2). A large percentage of cells from the three environmental isolates were SYTOX permeable. Specifically, all of the strains were more than 99% permeable at 48 h. These results show that the environmental isolates have decreased cell membrane integrity relative to that of MG1655 during fermentation, which indicates that the lower fatty acid titer achieved by MG1655 cannot be attributed to a loss of membrane integrity.

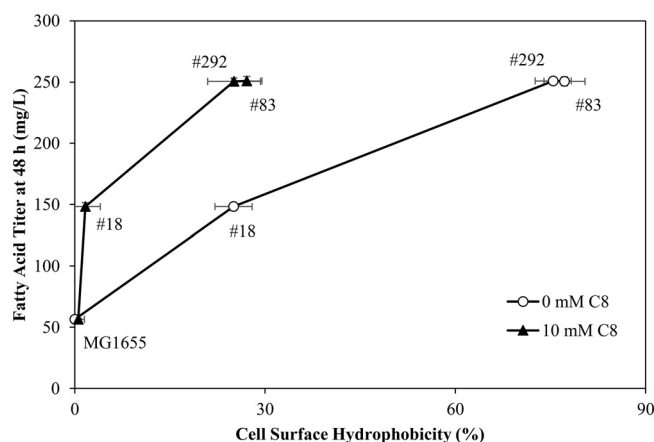


FIG 4 Increased fatty acid production is associated with higher cell surface hydrophobicity. Cell surface hydrophobicity was measured for mid-log cells growing in minimal medium with 0 mM or 10 mM C_8 at 37°C, and fatty acid titers are reported in Table 2. All values are the average of three biological replicates with error bars indicating one standard deviation.

Higher cell surface hydrophobicity leads to higher fatty acid production. The measurements of membrane polarization and integrity described above reflect the physical state of the phospholipid bilayer. Contrastingly, measurement of the cell surface hydrophobicity reflects the abundance and type of proteins and sugars on the cell surface, distinct from the membrane lipid distribution and associated fluidity (47). The environmental isolates used in this study are part of a larger set that was previously characterized in terms of various cell surface properties, including hydrophobicity (41). Hydrophobicity was found to range from 1 to 90% adhesion to hydrocarbons (41). Thus, in the current work, we also characterized the cell surface hydrophobicity during fatty acid challenge. The three focal environmental isolates all had increased hydrophobicity relative to that of MG1655, with and without exogenous C_8 challenge in MOPS minimal medium at 30°C (Fig. 4). Also, each of the environmental isolates showed a decrease in hydrophobicity during C_8 challenge relative to that under the control condition. Thus, the environmental isolates differ from MG1655 in both the value of the hydrophobicity and its modulation in response to C_8 .

A positive correlation between the cell surface hydrophobicity and fatty acid production was observed (Fig. 4). Specifically, fatty acid titers increased as cell hydrophobicity increased. These results suggest that increasing the cell surface hydrophobicity might be a strategy for improving fatty acid production. It has been previously demonstrated in yeast that increasing the cell surface hydrophobicity increased the tolerance of nonane (48) and increased the production of ethanol in the presence of inhibitors, such as carboxylic acids, furfural, and biomass hydrolysate (49).

Expression tuning of outer membrane protein BhsA in *E. coli* was previously demonstrated to change the cell surface hydrophobicity (50). Here, we altered the cell surface hydrophobicity of the four focal strains by expressing *bhsA* via the pACYC184 plasmid with the MG1655 *bhsA* promoter. For strain MG1655 and isolate 18, the cell surface hydrophobicity significantly ($P < 0.05$) increased when *bhsA* expression was increased in either the absence or the presence of C_8 (Fig. 5). The expression of *bhsA* had no impact on the hydrophobicity of strain 83 in the absence of C_8 but decreased hydrophobicity in the presence of C_8 . In contrast, for isolate 292, *bhsA* expression decreased hydrophobicity only in the absence of C_8 . Thus, in accordance with previous reports, perturbation of *bhsA* expression can alter the cell surface hydrophobicity of *E. coli* strains. However, the trends were not consistent across strains and conditions.

This perturbation of the hydrophobicity was performed in order to investigate the relationship between cell surface hydrophobicity and fatty acid production. Consistent with the increase in hydrophobicity for the MG1655 *bhsA*⁺ and 18 *bhsA*⁺ strains, fatty acid titers also significantly increased (Fig. 5B). The association of hydrophobicity and

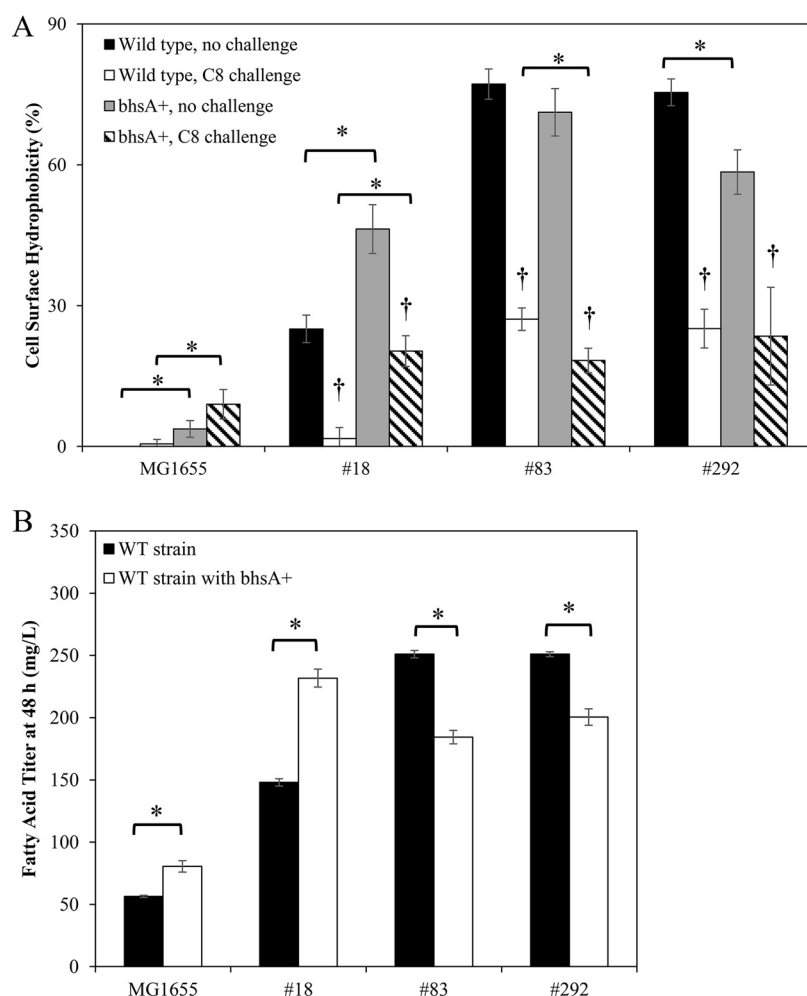


FIG 5 Expression of *bhsA* alters cell surface hydrophobicity and fatty acid production. Values are the average of three biological replicates, with error bars indicating one standard deviation. (A) Cell surface hydrophobicity with and without exogenously provided C_8 . Cells were grown in MOPS minimal medium at 37°C with and without 10 mM C_8 , with an initial pH of 7.0. (B) Fatty acid production via expression of the *A. tetradis* thioesterase in MOPS minimal medium at 30°C with an initial pH of 7.0. †, significant difference ($P \leq 0.05$) in the cell surface hydrophobicity between the two indicated conditions for the same strain. *, significant difference ($P \leq 0.05$) in the cell surface hydrophobicity between wild-type strain and the *bhsA*⁺ strain for the same condition.

fatty acid production was conserved for the isolates 83 and 292. Specifically, these strains each showed a decrease in cell surface hydrophobicity, either in the presence or absence of C_8 , and they each showed a significant decrease in fatty acid titer. Thus, tuning of cell surface hydrophobicity is a promising method of improving fatty acid production, but additional genetic targets for altering hydrophobicity in *E. coli* are needed.

DISCUSSION

Implementation of the design-build-test-learn cycle (22) in the construction of improved fermentation organisms can benefit from the characterization of evolved or nonmodel organisms (18, 20, 51–53). The environmental isolates characterized here were able to produce fatty acids at a substantially higher titer (up to 250 mg/liter) than the corresponding lab strain MG1655 in minimal medium (Table 2). Engineering of *E. coli* lab strains, such as MG1655 and BL21, to produce between 200 and 275 mg/liter of short-chain fatty acids in minimal medium has been described (40, 54, 55). Multiple genetic modifications of MG1655, combined with medium optimization and fed-batch operation, have even enabled the production of up to 1 g/liter octanoic acid (17). Thus,

while the fatty acid titers achieved by these environmental isolates do not surpass existing reports, it should be emphasized that these titers were observed in the absence of any genetic modifications beyond expression of the thioesterase.

We found that several aspects of the *E. coli* membrane, such as its composition and physical properties, were associated with increased tolerance to and production of octanoic acid. The ability to modulate the relative abundance of unsaturated lipids and the resulting impact on tolerance phenotypes have been previously reported in *E. coli* and *Saccharomyces cerevisiae* (33, 43, 56). Here, we saw that the high-producing environmental isolates did have an altered abundance of unsaturated lipids compared to MG1655 (Table 3). Additionally, our results suggest that the $C_{16:1}/C_{16:0}$ ratio may be a useful metric distinct from the standard consideration of total pools of saturated and unsaturated lipids. However, the observed differences in membrane fluidity (Fig. 3A) did not appear to be solely attributable to the relative abundances of unsaturated lipids.

Engineering of the average lipid length has also been previously reported as a strategy for increasing fatty acid tolerance and production in *E. coli* (39), and an evolved *E. coli* strain with increased fatty acid tolerance and production also showed an increase in average lipid length (18). Each of the environmental isolates characterized here showed a decrease in the average lipid length during fatty acid production relative to that under the control condition (Table 3). Thus, an increase in average lipid length is not required for attainment of the high fatty acid titers achieved by these isolates, possibly due to other differences in membrane composition.

The isolates characterized here show intriguing changes in the relative abundances of cyclopropane fatty acids (Table 3). The relative abundance of these molecules has been previously reported as impacting *E. coli* tolerance of various stressors, such as heat, pressure, and acid stress (57, 58). However, previous attempts to increase the abundances of these fatty acids in order to increase the tolerance of fatty acids have not been successful (9, 10).

The overall membrane composition, not just in terms of the fatty acid tails, but other components, such as proteins, phospholipid head groups, and polysaccharides, contributes to the bulk membrane properties, such as fluidity, integrity, rigidity, curvature, and thickness (59–61). Our characterization of environmental isolates with increased fatty acid production titers supports the idea that membrane fluidity is a critical determinant of fatty acid production capability (Fig. 3A), consistent with previous reports that decreased fluidity is associated with increased production (16, 18). However, the substantial loss of membrane integrity during the course of fatty acid production by the environmental isolates (Fig. 3B) does not appear to be detrimental to their ability to achieve high fatty acid titers. This suggests that focusing on engineering of membrane fluidity may be more impactful than trying to combat the decrease in membrane integrity often observed during bioproduction.

Distinct from the physical properties of the membrane lipid bilayer, the proteins and sugars associated with the membrane contribute to cell surface properties, such as hydrophobicity and charge (41, 61). Changes in bacterial hydrophobicity via alterations in the abundances of certain proteins and sugars have previously been reported to affect the tolerance of fatty acids (62, 63) and antibiotics (61, 64). Our environmental isolate characterization indicates that cell surface hydrophobicity is strongly associated with the ability to produce fatty acids at a high titer (Fig. 4). Thus, engineering strategies that enable tuning of cell surface hydrophobicity may be useful for improving bioproduction.

Consistent with previous reports (50), the expression of *bhsA* from a plasmid was shown to impact cell surface hydrophobicity (Fig. 5A) in a manner that was consistent with changes in fatty acid titers (Fig. 5B). However, the impact of this perturbation of BhsA expression on cell surface hydrophobicity varied according to the strain background, and in some cases, the change was relatively small. This variability in the effect of plasmid-based alteration of *bhsA* expression may be due to differences in the basal *bhsA* expression levels among isolates or differences in the expression of the plasmid-based gene

copy. Additionally, the baseline variation in cell surface hydrophobicity among these strains may be due to differences in abundance or sequence of membrane-associated proteins and enzymes that produce membrane-associated sugars.

It has been previously demonstrated that decreasing cell surface hydrophobicity decreases the interaction of fatty acids, such as decanoic acid, with the cell membrane (62). However, here, we observed that increasing hydrophobicity was associated with increased fatty acid production (Fig. 4). Decreasing cell surface hydrophobicity may be an effective strategy for preventing the entry of exogenous fatty acids (62), but this strategy may prove harmful to cells that are producing fatty acids endogenously. It is also possible that the high hydrophobicity values observed for the environmental isolates are due to the expression of a particular protein or sugar that is helpful in the tolerance and production of fatty acids. Thus, additional strategies for understanding the factors that contribute to cell surface hydrophobicity, as well as the ability to predictably and substantially change cell surface hydrophobicity, could be useful for the strain design toolkit.

MATERIALS AND METHODS

Strains, plasmids, and bacterial cultivation. All bacterial strains and plasmids used in this study are listed in Table 1. The collection and characterization of the environmental isolates were described elsewhere (41, 65, 66). These strains have not been sequenced but are available upon request. *E. coli* strains were grown overnight in 250-ml flasks with 10 ml of MOPS medium with 2.0 wt% dextrose at pH 7.00 \pm 0.05, at 37°C and 250 rpm. For octanoic acid tolerance tests, the overnight seed culture was diluted to an optical density at 550 nm (OD₅₅₀) of 0.05. For fatty acid fermentation, measurement of membrane leakage, membrane fluidity, cell hydrophobicity, and cell membrane composition, the overnight seed culture was diluted to an OD₅₅₀ of 0.1. Ampicillin (100 mg/liter) and kanamycin (50 mg/liter) were added as needed. All growth media and phosphate-buffered saline solutions had an initial pH of 7 \pm 0.05.

Octanoic acid tolerance test. Tolerance to octanoic acid was assessed in 250-ml baffled flasks with 25 ml MOPS medium with 2.0 wt% dextrose with or without exogenous 10 mM C₈, at 37°C and 200 rpm. Octanoic acid was provided via a stock solution containing 4.0 M C₈ in ethanol. The pH of the medium was adjusted using 2.0 M KOH.

Fatty acid production. Strains were transformed with the pJMY-EEI82564 plasmid (18) by electroporation and grown on LB plates with ampicillin at 30°C overnight. Individual colonies were inoculated in 10 ml MOPS medium with 2.0 wt% dextrose and ampicillin in 250-ml flasks at 30°C on a rotary shaker at 250 rpm overnight. These seed cultures were subsequently inoculated into 250-ml flasks containing 50 ml of MOPS medium with 2.0 wt% dextrose, ampicillin, and 1.0 mM isopropyl- β -D-thiogalactopyranoside (IPTG) at an initial OD₅₅₀ of 0.1. The flasks were incubated in a rotary shaker at 200 rpm and 30°C.

Determination of fatty acid titers. Fatty acid production was quantified by fatty acid extraction from samples containing both medium and cells. The extracted fatty acids were derivatized and measured at the ISU W. M. Keck Metabolomics Research Laboratory via an Agilent 6890 gas chromatograph coupled to an Agilent 5973 mass spectrometer (GC-MS), as previously described (55). Briefly, 1 ml of culture was transferred into a 2-ml microcentrifuge tube, and 125 μ l 10% (wt/vol) NaCl, 125 μ l acetic acid, 20 μ l internal standard (1 μ g/ μ l C₇, C₁₁, and C₁₅ in ethyl alcohol [EtOH]), and 500 μ l ethyl acetate were added sequentially. The mixture was vortexed for 30 s and centrifuged at 16,000 \times g at room temperature for 10 min. Two hundred fifty microliters of the top layer was transferred into a glass tube, and 2.25 ml of 30:1 EtOH–37% (vol/vol) HCl was added. This mixture was then incubated at 55°C for 1 h and then cooled to room temperature. Then, 1.25 ml each of double-distilled water (ddH₂O) and hexane was added, followed by vortexing and centrifugation at 2,000 \times g for 2 min. The top hexane layer was then analyzed by GC-MS using the following program: the initial temperature was 50°C, followed by a hold for 1 min, and then a temperature ramp of 20°C/min to 140°C, 4°C/min to 220°C, and 5°C/min to 280°C with 1 ml/min helium carrier gas.

The Enhanced Data Analysis program (Agilent Technologies) and NIST 17 Mass Spectral Library software were used for peak identification. The relative retention factors of C₇, C₁₁, and C₁₅ were used to calculate the relative amounts of the individual fatty acids analyzed.

Membrane lipid composition. Seed cultures were inoculated into 250-ml baffled flasks with 50 ml MOPS medium with 2.0 wt% dextrose at 30°C and 250 rpm. For analysis of the C₈ challenge, *E. coli* cells were harvested at mid-log phase (OD₆₀₀ \approx 0.8), resuspended in 25 ml medium with 0 mM or 30 mM C₈ at pH 7.0, and incubated for 3 h at 30°C. For analysis of C₈ production, cells were harvested at 24 and 48 h.

The harvested cells were washed twice in cold sterile water, resuspended into 6 ml methanol, and sonicated for three 30-s bursts. Then, 1.4 ml of this sonicated solution was transferred into each of three glass tubes to be processed in parallel (67). Twenty microliters of a solution containing 1 μ g/ μ l each of C₇, C₁₁, and C₁₅ in methanol was added. These mixtures were incubated at 70°C for 15 min and cooled to room temperature. The cells were centrifuged at 4,000 \times g for 5 min, and the resulting supernatant and pellet were treated separately. The supernatant was transferred into a new glass tube with 1.4 ml Nanopure water and vortexed. Seven hundred fifty microliters of chloroform was added to the pellet,

followed by vortexing and shaking in a horizontal shaker at 150 rpm and 37°C for 5 min. The diluted supernatant was added back to the chloroform-treated pellet. This mixture was vortexed for 2 min and then centrifuged at $3,000 \times g$ for 5 min. The bottom layer was transferred to a new glass tube and all solvent removed with an N-Evap nitrogen tree evaporator. Two milliliters of 1.0 N HCl in methanol was added to the dried samples, heated at 80°C for 30 min, and then cooled to room temperature. Two milliliters of 0.9 wt% aqueous NaCl and 1.0 ml hexane was added, followed by vortexing for 2 min and centrifugation at $2,000 \times g$ for 2 min. The upper layer was analyzed by GC, using the method described above.

The weight-average lipid length was calculated as previously described (9). The percent abundance of unsaturated fatty acids was calculated as the sum of the percent abundance of $C_{12:1}$, $C_{14:1}$, $C_{16:1}$, and $C_{18:1}$.

Membrane permeability. Cells were harvested at 24 h and 48 h. Following centrifugation at $4,500 \times g$ and 4°C for 10 min, cell pellets were washed twice with phosphate-buffered saline (PBS) and resuspended in PBS at a final OD_{550} of ~ 1 . Then, 100 μ l of resuspended cells was diluted with 900 μ l PBS. Cells were stained with 1 μ l of 5 mM SYTOX Green in dimethyl sulfoxide (Invitrogen, Carlsbad, CA), for a final concentration of 5 μ M. Cells were then analyzed by flow cytometry at the ISU Flow Cytometry Facility on a BD Biosciences FACSCanto II system (18). About 18,000 events were tested per sample, and each sample had three parallel groups.

Membrane fluidity. Membrane fluorescence polarization was measured using 1,6-diphenyl-1,3,5-hexatriene (DPH), as previously described (9, 46). In this assay, the orientation of this rod-shaped hydrophobic reporter molecule is indicative of the membrane fluidity (46). Harvested cells were prepared as described above for the assessment of membrane permeability. Five hundred microliters of cells in PBS at an OD_{550} of ~ 1 was transferred to a 1.5-ml centrifuge tube containing 500 μ l of 0.4 μ M DPH in PBS. The mixture was vortexed and incubated at 37°C in the dark for 30 min. The samples were then centrifuged ($5,000 \times g$, 5 min), and the cell pellets were resuspended in 500 μ l PBS. One hundred microliters of this mixture was transferred into sterile black-bottom Nunclon Delta surface 96-well plates with 4 replicates. A suspension of cells with no DPH was used as a control. Membrane fluorescence polarization values were determined based on vertical and horizontal fluorescence readings, which were assessed by via the BioTek Synergy 2 multimode microplate reader at the ISU W. M. Keck Metabolomics Research Laboratory.

Cell surface hydrophobicity. The method for measuring cell surface hydrophobicity was based on a previously described procedure (41, 68, 69). The seed culture was inoculated into 25 ml MOPS medium with 2 wt% dextrose with or without exogenous 10 mM C_8 at 30°C with rotary shaking at 250 rpm. Cells were harvested at mid-log phase (OD_{550} , ≈ 0.8), followed by centrifugation at $4,500 \times g$ and room temperature for 10 min. Cell pellets were then washed twice with PBS buffer and resuspended in PBS at a final OD_{550} of ~ 0.6 . Four milliliters of cell suspension was added to a glass tube, and the OD_{550} was measured (OD_1). Then, 1.0 ml of dodecane was added, and the mixture was mixed using a multitube vortexer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 2,500 rpm for 10 min. The mixtures were left to settle for 15 min at room temperature, and the OD_{550} of the aqueous phase (OD_2) was measured. Partitioning of the bacteria suspension was calculated using the following equation: percent partitioning = $[(OD_1 - OD_2)/OD_1] \times 100$.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.01285-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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